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Identification of One Novel Candidate Probiotic *Lactobacillus plantarum* Strain Active against Influenza Virus Infection in Mice by a Large-Scale Screening

Noura Kechaou,[a,b] Florian Chain,[a,b] Jean-Jacques Gratadoux,[a,b] Sébastien Blugeon,[a,b] Nicolas Bertho,[c] Christophe Chevalier,[c] Ronan Le Goffic,[c] Stéphanie Courau,[d] Pascal Molimard,[d] Jean Marc Chatel,[a,b] Philippe Langella,[a,b] Luis G. Bermúdez-Humarán[a,b]

INRA, Commensal and Probiotics-Host Interactions Laboratory, UMR1319 Micalis, Jouy-en-Josas, France[a]; AgroParisTech, UMR1319 Micalis, Jouy-en-Josas, France[b]; Unité de Virologie et Immunologie Moléculaires, UR892, Jouy-en-Josas, France[c]; Merck Médication Familiale, Dijon, France[d]

In this study, we developed a large-scale screening of bacterial strains in order to identify novel candidate probiotics with immunomodulatory properties. For this, 158 strains, including a majority of lactic acid bacteria (LAB), were screened by two different cellular models: tumor necrosis factor alpha (TNF-α)-activated HT-29 cells and peripheral blood mononuclear cells (PBMCs). Different strains responsive to both models (pro- and anti-inflammatory strains) were selected, and their protective effects were tested *in vivo* in a murine model of influenza virus infection. Daily intragastric administrations during 10 days before and 10 days after viral challenge (100 PFU of influenza virus H1N1 strain A Puerto Rico/8/1934 [A/PR8/34]/mouse) of *Lactobacillus plantarum* CNRZ1997, one potentially proinflammatory probiotic strain, led to a significant improvement in mouse health by reducing weight loss, alleviating clinical symptoms, and inhibiting significantly virus proliferation in lungs. In conclusion, in this study, we have combined two cellular models to allow the screening of a large number of LAB for their immunomodulatory properties. Moreover, we identified a novel candidate probiotic strain, *L. plantarum* CNRZ1997, active against influenza virus infection in mice.

Probiotics are defined by the Food and Agriculture Organization (FAO) of the United Nations World Health Organization (WHO) as "live microorganisms which, when administered in adequate amounts, confer health benefits on the host" (1). Their use is more and more popular for both prevention and treatment of a number of human diseases (2–5). Most probiotic microorganisms belong to lactic acid bacteria (LAB). However, not all probiotics display the same properties, and careful selection of specific strains based on their claimed beneficial effects is needed. Although several criteria have been proposed to identify novel probiotic strains, some studies have reported the selection of potential candidates at the preclinical level (i.e., animal trials), including evaluation of both safety and potential efficacy (6–8). The efficacy of probiotic bacteria is greatly influenced by their functional properties, such as antimicrobial activity, persistence in the gastrointestinal tract (GIT) for intestine-targeted probiotics, and immunomodulatory properties (9, 10).

Despite advances in medicine, common respiratory virus infections (RVI) such as the common cold or flu continue to cause a considerable economic burden; fortunately, some probiotic strains have been studied for their positive effects on these virus infections (11, 12). Indeed, products containing probiotics have been shown to have an immunomodulatory effect and a protective effect against RVI in both mice and humans (13–15). For example, oral daily administration of the LAB *Lactobacillus plantarum* L-137 (a strain selected for its proinflammatory properties *in vitro*) before and after influenza virus H1N1 challenge in mice enhanced survival and decreased virus titers in lungs of infected mice (15). Furthermore, several other LAB strains, such as *Lactobacillus fermentum* CECT5716 or *Lactobacillus casei* DN114-001, were described recently to enhance the effects of influenza virus vaccination and to improve antibody responses to influenza virus vaccination in humans, respectively (16, 17). Also, a mixture of *Lactobacillus gasseri* PA 16/8, *Bifidobacterium longum* SP 07/3, and *Bifidobacterium bifidum* MF 20/5 reduced the severity of symptoms related to common cold episodes in humans (14). In another clinical trial, the most commonly used probiotic strain, *Lactobacillus rhamnosus* GG, tested alone or in association with *B. animalis* subsp. *lactis* BB-12, reduced the incidence of RVI (18, 19). A clinical trial using *Lactobacillus acidophilus* strain NCFM alone or in association with *Bifidobacterium animalis* subsp. *lactis* BI-07 showed that these probiotics reduce influenza-like symptoms (fever, rhinorrhea, cough incidence, and duration of antibiotic prescription) (20). In addition, in another interesting study, Boge et al. (16) demonstrated that daily consumption of a probiotic fermented dairy drink improves antibody responses to influenza virus vaccination in the elderly in two randomized controlled trials. Altogether, these preclinical and clinical trials suggest that probiotics can be successfully used as preventive and therapeutic agents in RVI.

Over the last 5 years, the interest in the immunomodulatory properties of LAB strains has significantly increased. In this context, a wide variety of strain-dependent properties have been reported (8, 10, 13), and several *in vitro* cellular models have been developed in order to analyze and classify the immunomodulatory properties of these strains. The aim of this study is thus to determine the immunomodulatory properties of a large set of LAB (158 strains) using two different cellular models, tumor necrosis

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Address correspondence to Philippe Langella, philippe.langella@jouy.inra.fr, or Luis G. Bermúdez-Humarán, luis.bermudez@jouy.inra.fr.
N.K. and F.C. contributed equally.

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TABLE 1 Strains used in this study

Organism	Collection no.	Origin
Lactobacillus delbrueckii	VEL12191	Yogurt
Lactobacillus delbrueckii	VEL12192	Yogurt
Lactobacillus helveticus	VEL12193	Yogurt
Lactobacillus paracasei	VEL12194	Human tooth decay
Lactobacillus paracasei	VEL12195	Vegetable
Lactobacillus plantarum	VEL12196	Unknown
Lactobacillus plantarum	VEL12197	Fermented vegetable
Lactobacillus rhamnosus	VEL12198	Unknown
Lactobacillus pentosus	VEL12199	Corn silage
Lactobacillus pentosus	VEL12200	Cheese
Lactobacillus johnsonii	VEL12201	Unknown
Lactobacillus johnsonii	VEL12202	Human blood
Lactobacillus johnsonii	VEL12203	Unknown
Lactobacillus casei	VEL12204	Cheese
Lactobacillus casei	VEL12205	Cheese
Lactobacillus casei	VEL12206	Unknown
Lactobacillus casei	VEL12207	Cheese
Lactobacillus brevis	VEL12208	Healthy donor
Lactobacillus fermentum	VEL12209	Human saliva
Lactobacillus fermentum	VEL12210	Fermented vegetable
Lactobacillus zeae	VEL12211	Vegetable
Lactobacillus zeae	VEL12212	Vegetable
Lactobacillus intestinalis	VEL12213	Animal bowels
Lactobacillus crispatus	VEL12214	Healthy donor
Lactobacillus crispatus	VEL12215	Human eyes
Lactobacillus crispatus	VEL12216	Chicken cecum
Lactobacillus gasseri	VEL12217	Human saliva
Lactobacillus gasseri	VEL12218	Human tooth decay
Lactobacillus salivarius	VEL12219	Human saliva
Lactobacillus salivarius	VEL12220	Human saliva
Lactobacillus sakei	VEL12221	Fermented drink
Lactobacillus reuteri	VEL12222	Healthy donor
Lactobacillus reuteri	VEL12223	Healthy donor
Lactobacillus curvatus	VEL12224	Meat
Lactobacillus curvatus	VEL12225	Meat
Lactobacillus curvatus	VEL12226	Unknown
Lactobacillus delbrueckii subsp. *lactis*	VEL12227	Cheese
Lactobacillus delbrueckii subsp. *lactis*	VEL12228	Cheese
Lactobacillus acidophilus	VEL12229	Unknown
Lactobacillus helveticus	VEL12230	Cheese
Lactobacillus rhamnosus	VEL12231	Unknown
Lactobacillus brevis	VEL12232	Unknown
Lactobacillus lactis	VEL12233	Unknown
Lactobacillus johnsonii	VEL12234	Unknown
Lactobacillus johnsonii	VEL12235	Cheese
Lactobacillus delbrueckii subsp. *bulgaricus*	VEL12236	Yogurt
Lactobacillus paracasei subsp. *paracasei*	VEL12237	Fermented vegetable
Lactobacillus plantarum	VEL12238	Yak milk
Lactobacillus plantarum	VEL12239	Bakery starter
Lactobacillus paracasei subsp. *paracasei*	VEL12240	Fermented vegetable
Lactobacillus plantarum	VEL12241	Fermented vegetable
Lactobacillus casei BL23 MnKat$^-$	VEL11757	Recombinant strain
Lactobacillus casei BL23 MnKat$^+$	VEL11758	Recombinant strain
Lactobacillus plantarum 8826	VEL11685	Human saliva
Lactobacillus acidophilus NCFM	VEL12085	Human donor
Lactobacillus casei DN114-001	VEL12079	Commercial strain
Lactobacillus salivarius Ls33	VEL12093	
Lactobacillus casei BL23	VEL12016	Milk

TABLE 1 (Continued)

Organism	Collection no.	Origin
Lactobacillus bulgaricus	VEL12374	Unknown
Lactobacillus brevis	VEL12348	Unknown
Lactobacillus bulgaricus	VEL12349	Unknown
Lactobacillus bulgaricus	VEL12350	Milk
Lactobacillus bulgaricus	VEL12351	Unknown
Lactobacillus crispatus	VEL12352	Unknown
Lactobacillus crispatus	VEL12353	Unknown
Lactobacillus fermentum	VEL12354	Unknown
Lactobacillus gasseri	VEL12355	Unknown
Lactobacillus helveticus	VEL12356	Unknown
Lactobacillus lactis	VEL12357	Cheese
Lactobacillus lactis	VEL12358	Yak milk
Lactobacillus plantarum	VEL12359	Unknown
Lactobacillus paraplantarum	VEL12360	Commercial starter
Lactobacillus paracasei	VEL12361	Fermented milk
Lactobacillus paracasei	VEL12362	Unknown
Lactobacillus rhamnosus	VEL12363	Unknown
Lactobacillus salivarius	VEL12364	Commercial starter
Lactobacillus salivarius	VEL12365	Unknown
Lactobacillus sakei	VEL12366	Unknown
Lactobacillus rhamnosus	VEL12386	Unknown
Lactobacillus vaginalis	VEL12367	Unknown
Lactobacillus delbrueckii	VEL12377	Unknown
Lactobacillus delbrueckii	VEL12378	Unknown
Lactobacillus delbrueckii	VEL12379	Unknown
Lactobacillus delbrueckii	VEL12380	Unknown
Lactobacillus delbrueckii	VEL12381	Unknown
Lactobacillus delbrueckii	VEL12382	Unknown
Lactobacillus delbrueckii	VEL12383	Unknown
Lactobacillus delbrueckii	VEL12384	Unknown
Lactobacillus fermentum	VEL12385	Unknown
Lactococcus lactis subsp. *cremoris*	VEL12242	Unknown
Lactococcus lactis subsp. *cremoris*	VEL12243	Cheese
Lactococcus lactis subsp. *lactis*	VEL12244	Cheese
Lactococcus lactis subsp. *lactis*	VEL12245	Unknown
Lactococcus lactis subsp. *lactis*	VEL12246	Unknown
Lactococcus lactis subsp. *lactis*	VEL12247	Unknown
Lactococcus lactis subsp. *lactis*	VEL12248	Unknown
Lactococcus lactis subsp. *lactis*	VEL12249	Cheese
Lactococcus lactis subsp. *lactis*	VEL12250	Unknown
Lactococcus lactis subsp. *lactis*	VEL12251	Fermented milk
Lactococcus raffinolactis	VEL12252	Milk
Lactococcus lactis subsp. *lactis*	VEL12253	Milk
Lactococcus lactis subsp. *lactis*	VEL12254	Unknown
Lactococcus lactis subsp. *cremoris*	VEL12255	Unknown
Lactococcus lactis subsp. *cremoris*	VEL12256	Unknown
Lactococcus lactis subsp. *lactis*	VEL12257	Unknown
Lactococcus garvieae	VEL12258	Unknown
Lactococcus plantarum	VEL12259	Unknown
Lactococcus lactis subsp. *cremoris*	VEL12260	Unknown
Lactococcus lactis subsp. *cremoris*	VEL12261	Unknown
Lactococcus lactis subsp. *lactis*	VEL12262	Yak milk
Lactococcus lactis subsp. *hordniae*	VEL12263	Unknown
Lactococcus lactis subsp. *cremoris*	VEL12264	Unknown
Lactococcus lactis subsp. *cremoris*	VEL12265	Cheese
Lactococcus lactis subsp. *lactis*	VEL12266	Cheese starter
Lactococcus lactis subsp. *lactis*	VEL12267	Cheese
Lactococcus lactis subsp. *cremoris* NCDO712	J60011	Recombinant strain
Spontaneous mutant *Lactococcus lactis* subsp. *cremoris* NCDO712	Spox K	Recombinant strain

(Continued on following page)

TABLE 1 (Continued)

Organism	Collection no.	Origin
Lactococcus lactis subsp. *cremoris* NCDO712	MG1363	Milk
Lactococcus lactis SL106	VEL12347	Unknown
Lactococcus lactis TIL46	VEL12375	Unknown
Bifidobacterium longum	VEL12268	Unknown
Bifidobacterium thermophilum	VEL12269	Rumen bovine
Bifidobacterium longum	VEL12270	Fermented drink
Bifidobacterium adolescentis	VEL12271	Healthy donor
Bifidobacterium thermophilum	VEL12272	Rumen bovine
Bifidobacterium longum	VEL12273	Fermented drink
Bifidobacterium longum	VEL12274	Fermented drink
Bifidobacterium bifidum	VEL12275	Healthy donor
Bifidobacterium breve	VEL12276	Healthy donor
Bifidobacterium adolescentis	VEL12277	Healthy donor
Bifidobacterium bifidum	VEL12328	Healthy donor
Bifidobacterium bifidum	VEL12329	Healthy donor
Bifidobacterium sp.	VEL12330	Healthy donor
Bifidobacterium sp.	VEL12331	Healthy donor
Bifidobacterium sp.	VEL12332	Healthy donor
Bifidobacterium sp.	VEL12333	Healthy donor
Bifidobacterium sp.	VEL12334	Healthy donor
Bifidobacterium angulatum	VEL12335	Unknown
Bifidobacterium adolescentis	VEL12336	Unknown
Bifidobacterium pseudocatenulatum	VEL12337	Unknown
Bifidobacterium longum	VEL13385	Unknown
Bifidobacterium longum subsp. *animalis*	VEL12338	Unknown
Bifidobacterium angulatum	VEL12339	Unknown
Bifidobacterium infantis	VEL12340	Unknown
Bifidobacterium animalis	VEL12341	Unknown
Bifidobacterium sp.	VEL12342	Healthy donor
Bifidobacterium sp.	VEL12343	Healthy donor
Bifidobacterium sp.	VEL12344	Healthy donor
Bifidobacterium sp.	VEL12345	Healthy donor
Bifidobacterium sp.	VEL12346	Healthy donor
Bifidobacterium bifidum CIP 56.7 (ATCC 29251)	VEL12376	Unknown
Streptococcus thermophilus	VEL12368	Unknown
Streptococcus thermophilus	VEL12369	Unknown
Pediococcus pentosaceus	VEL12370	Unknown
Pediococcus acidilactici	VEL12371	Unknown
Streptococcus thermophilus	VEL12372	Unknown
Bacillus subtilis	VEL12373	Unknown
Lactobacillus delbrueckii	VEL12387	Unknown

factor alpha (TNF-α)-activated HT-29 cells and peripheral blood mononuclear cells (PBMCs), in order to identify the most efficient strain and develop a new probiotic supplement able to alleviate symptoms of RVI and, more particularly, the common cold. After establishing the immunomodulatory profile (based on their cytokine production profile) of each strain of the collection, different strains responsive to both models (pro- and anti-inflammatory strains) were selected and tested *in vivo* in a murine model of RVI, an influenza virus infection, to determine their protective effects.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The collection of this study comprised 158 strains isolated from different biotopes and including a majority of LAB (90 *Lactobacillus* spp., 31 *Lactococcus* spp., 31 *Bifidobacterium* spp., 3 *Streptococcus* spp., 2 *Pediococcus* spp., and 1 *Bacillus* sp.; INRA Collection CIRM, Rennes, France) (Table 1). *Lactococcus* spp. and *Streptococcus* spp. were grown in M17 medium (Difco, KS) supplemented with 5% lactose at 30°C without shaking, *Lactobacillus* and *Pediococcus* spp. were grown in MRS medium (Difco, KS) at 37°C without shaking, *Bifidobacterium* spp. were grown in MRS medium supplemented with 10% cysteine (Sigma-Aldrich, France) at 37°C under anaerobic conditions (GENbox Microaer; bioMérieux, Marcy l'Etoile, France) without shaking, and *Bacillus* spp. were grown in Luria-Bertani medium (Difco, KS) at 37°C with shaking.

Bile salts stress resistance assessment. Resistance to bile salts was studied to mimic the passage of the strains in the GIT. A high-throughput method based on sterile microwell plates (384 wells; Greiner, Bio-one) was used. From a culture of each strain grown overnight, the optical density at 600 nm (OD_{600}) was adjusted to 1 in peptone water. Five hundred microliters from this adjusted culture was added either to 500 μl of a cholic acid (Sigma-Aldrich, France) and deoxycholic acid (Sigma-Aldrich, France) solution in peptone water (0.05% [vol/wt] for each acid) or to 500 μl of peptone water. After 1 h at 37°C, stressed and nonstressed bacterial suspensions were centrifuged. Pellets were resuspended in 1 ml of appropriate culture medium and diluted 10 times. Sixty microliters from those suspensions was deposited into a well already containing 60 μl of medium. Four serial 2-fold dilutions were then performed. Growth of the stressed and nonstressed strains was monitored every 15 min for 19 h by measuring the OD_{600} using a thermoregulated plate reader (InfiniteM200 Pro; Tecan, France). The resistance of each strain to bile salts stress was determined by measuring the growth delay (i.e., delay for the time to reach mid-exponential phase) between stressed and nonstressed cultures. For each strain, 5 growth delays (in each experiment) corresponding to the five dilutions on the microplate were averaged. Experiments were performed in triplicates.

Experiments with the HT-29 cell line. The human colon carcinoma cell line HT-29 was cultured in 24-well culture plates in Dulbecco's modified Eagle's medium (DMEM) (Lonza, Switzerland) supplemented with 10% heat-inactivated fetal calf serum (FCS)–1% glutamine at 37°C in a 10% CO_2–air atmosphere. Medium was changed every day. Experiments were initiated on day 7 after seeding, when cells were at confluence (~1.83×10^6 cells/well). Twenty-four hours before bacterial coculture (day 6), the culture medium was changed for a medium with 5% heat-inactivated FCS and 1% glutamine. On the day of coculture, bacteria were added at a multiplicity of infection (MOI) of 1:40 in 50 μl DMEM in a total volume of 500 μl. Cells were stimulated simultaneously with recombinant human TNF-α (5 ng/ml; Peprotech, NJ) for 6 h at 37°C in 10% CO_2. All samples were analyzed in triplicate. After coincubation, cell supernatants were collected and frozen at −80°C until further analysis of interleukin-8 (IL-8) concentrations by an enzyme-linked immunosorbent assay (ELISA) (Biolegend, San Diego, CA).

Experiments with PBMCs. Commercial PBMCs (StemCell Technologies SARL, Grenoble, France) from healthy donors were used. For the first screening (i.e., 158 strains), we used PBMCs from one healthy donor (American man, Caucasian, aged <65 years, with a body mass index of <30), nonsmoking, with no drugs with anti-inflammatory effects taken during 15 days prior to sampling and negative for HIV and hepatitis A and B viruses. For the confirmation phase, we used PBMCs from 3 different healthy donors presenting similar health characteristics. After reception, cells were stored in liquid nitrogen until use. To prepare PBMCs for coculture experiments with bacteria, the vial cells were thawed at 37°C in a water bath and then transferred into a medium containing RPMI 1640 medium (Lonza, Switzerland) supplemented with 10% heat-inactivated FCS, 1% L-glutamine, and 0.1% penicillin-streptavidin. DNase (10 mg/ml) was added to this mix to avoid clumping. Cells were then centrifuged at 200 × *g* for 15 min, counted by using trypan blue, and spread onto 24-well plates at 1×10^6 cells/well. Bacteria were added in triplicate at an MOI of 1:10 in 20 μl of phosphate-buffered saline (PBS) in a total volume of 1 ml, and plates were incubated for 24 h at 37°C in 10% CO_2. Samples

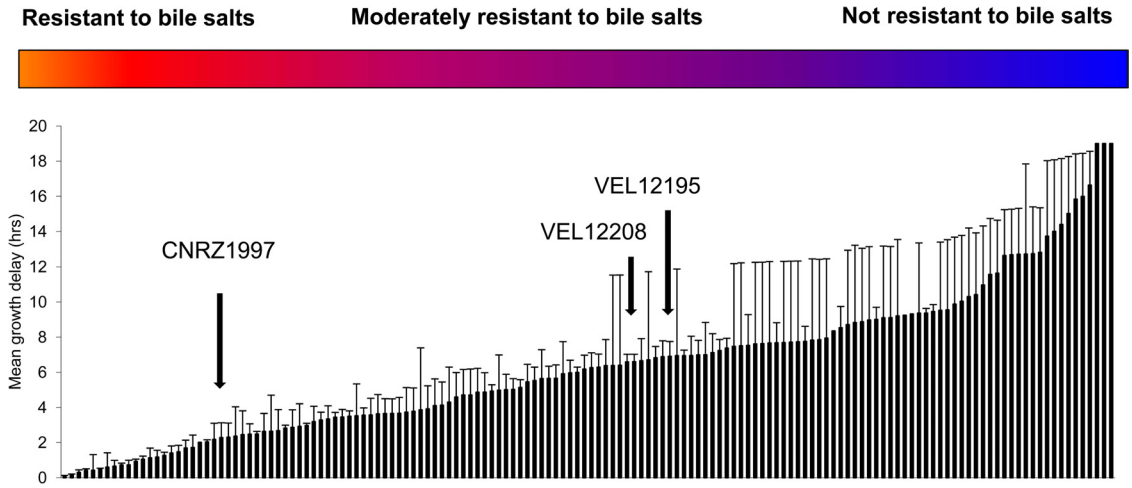

FIG 1 Classification of the bacterial strains according to their resistance to bile salts. Results are expressed as the mean delay in growth after exposure to the stress.

were finally stored at −80°C until further analysis of IL-10 and IL-12p70 concentrations by ELISA (Mabtech, Sweden).

Mice. Specific-pathogen-free BALB/c mice (females, 6 weeks of age; Janvier, France) were maintained under normal husbandry conditions in the animal facilities of the National Institute of Agricultural Research (UEAR, INRA, Jouy-en-Josas, France). All animal experiments were started after the animals were allowed 2 weeks of acclimation and were performed according to European Community rules of animal care and with authorization 78-149 of the French Veterinary Services.

Influenza virus infection of mice. Influenza virus H1N1 strain A Puerto Rico/8/1934 (A/PR8/34; a mouse-adapted strain) was grown in allantoic cavities of 11-day-old fertile chicken eggs for 2 days at 35°C (21). The viral titer (i.e., PFU determination) was quantified by a standard plaque assay using Madin-Darby canine kidney (MDCK) cells, and the virus stock was stored at −80°C until use. For intranasal infection, mice were fully anesthetized by intraperitoneal injection of ketamine (Imalgene 1000, Merial, France) and xylazine (Rompun, Alcyon, France) (0.1% ketamine plus 0.06% xylazine; 150 μl for a mouse weighing 20 g) and then infected by intranasal application of 50 μl of virus suspension (25 μl into each nostril).

Preparation of live bacterial inocula and administration in mice. Candidate probiotic bacteria were grown as described above. Pellets from cultures grown overnight were then harvested by centrifugation at 3,000 × *g* at 4°C and washed with sterile PBS. The pellet was suspended in PBS to a final concentration of 5×10^9 CFU/ml. Plate counts were performed with all inocula to corroborate the CFU administered. As a positive control for our *in vivo* experiments, we used *L. casei* DN114-001 and *L. rhamnosus* GG, two probiotic strains having well-documented *in vitro* and *in vivo* immunomodulatory properties and protective effects in different models of influenza virus infection, as positive controls (13, 16, 22, 23).

Groups of mice ($n = 8$) were daily administered intragastrically 1×10^9 CFU of each strain suspended in 200 μl of PBS (with a feeding needle) 10 days before and 10 or 14 days after virus challenge. PBS was used as a negative control. Mice were monitored daily for mortality, weight loss, and visual score by scientists blinded to the study. For visual score, we used the following five-point scale, as reported previously (24): 5, healthy (no clinical symptoms); 4, mild (fur slightly ruffled); 3, moderate (fur moderately ruffled and lethargy); 2, severe (fur severely ruffled and thin); 1, very severe (no reaction to stimulation).

Sample collection. On days 10 and 14 postinfection, blood samples were obtained from the retro-orbital venous plexus and centrifuged, and sera were stored at −80°C until further analysis. Mice were then sacrificed by cervical dislocation, and bronchoalveolar lavage fluid (BALF) was collected. To recover BALF, a catheter was tied to the exposed trachea, and a hypodermic needle and syringe were attached and used to inject and withdraw the lungs with a total volume of 1 ml of PBS. BALF samples were stored at −80°C until further analysis. Furthermore, lungs were collected for virus titration and stored at −80°C until analysis.

Virus quantification in mouse lungs. Total RNA was isolated by using a Qiagen RNeasy minikit (Qiagen, France). Reverse transcription was carried out with Superscript II reverse transcriptase (Invitrogen, France) and the specific influenza A virus (IAV) M1 primer (21), 5′-TCT AAC CGA GGT CGA AAC GTA-3′, according to the supplier's recommendations. Virus titers were quantified by quantitative PCR. We used the specific IAV M1 primer 5′-TCT AAC CGA GGT CGA AAC GTA-3′ and the Mastercycler Realplex system (Eppendorf, France). The PCR conditions and cycles were as follows: initial DNA denaturation for 10 min at 95°C followed by 40 cycles (15 s at 95°C, 20 s at 64°C, and 30 s at 72°C), followed by 15 s at 95°C, 15 s at 60°C, 20 min of melting curve (to assess the purity of the PCR product), and 15 s at 95°C. To normalize gene expression, β-actin levels were determined for all samples (sense primer 5′-AGA AAA TCT GGC ACC ACA CC-3′ and antisense primer 5′-CTC CTTAAT GTC ACG CAC GA-3′) (21).

Statistical analyses. For animal experiments, the formula used to calculate sample size was $n = 1 + 2 \times C \times (s/d)^2$ (where s is the standard deviation, d is the difference to be observed with a C value of 10.51 [for an alpha value of 0.05 and a beta value of 0.1], and s/d equals 0.5). With these values, n equals 6.255, which means a sample size of 7 animals (and we used an extra animal to take into account the fact that mice can sometimes escape virus infection). Data were analyzed by using Dunnett's test or *t* test to compare the differences between groups and controls using Prism software. A P value of <0.05 was considered significant.

RESULTS

***In vitro* screening of the strain collection.** Our bacterial strain collection was first analyzed in a stress resistance experiment. As shown in Fig. 1, 44% of the strains (70 strains) had a growth delay of less than 5 h and were qualified as resistant to bile salts. We then determined the immunomodulatory properties of the 158 strains in the two cellular models: TNF-α-stimulated HT-29 cells and PBMCs.

For the HT-29 model, we analyzed TNF-α-induced IL-8 secretion by HT-29 cells. Since IL-8 is considered a major inflammatory mediator, bacteria enhancing its secretion are considered to have proinflammatory properties, while those inhibiting its secretion are considered to have anti-inflammatory properties. As shown in Fig. 2, the 158 strains revealed a very distinct pattern of secretion of IL-8 in TNF-α-stimulated HT-29 cells and can be

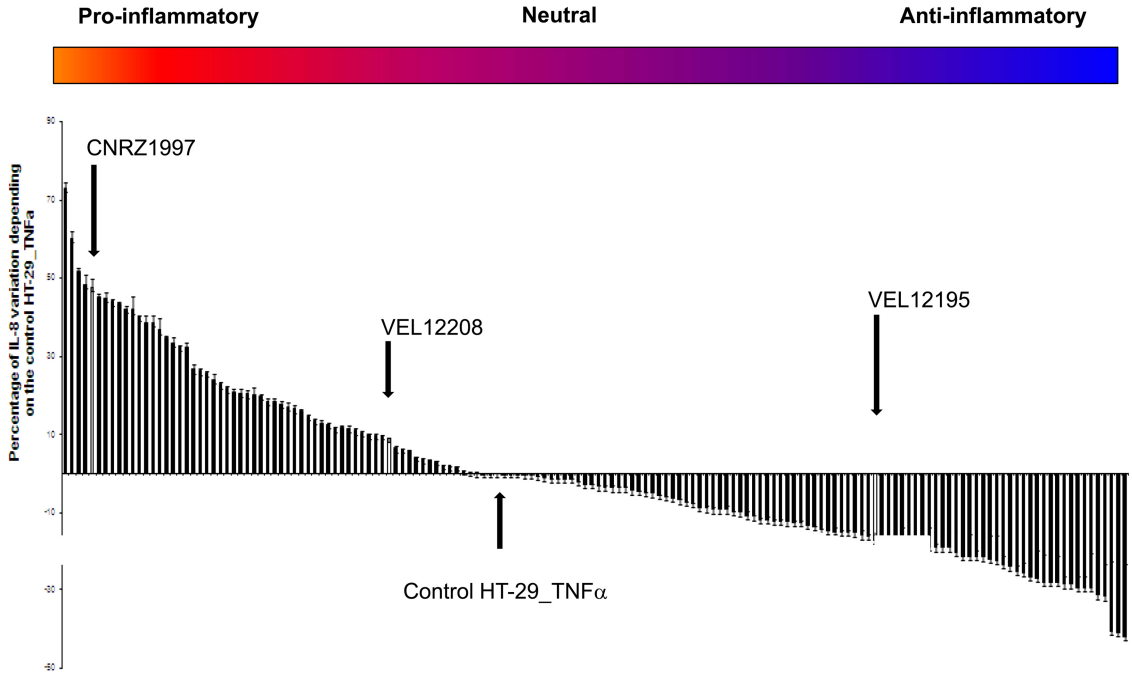

FIG 2 Classification of bacterial strains according to IL-8 production by HT-29 cells stimulated with TNF-α. Cytokine production after the coincubation of bacteria and HT-29 cells for 6 h was analyzed by ELISA. The results are expressed as a percentage of induction of the HT-29/TNF-α/PBS control ± standard error of the mean ($n = 3$).

classified as highly to weakly proinflammatory, including some neutral strains. None of the bacteria tested alone (i.e., nonstimulated HT-29 cells) induced IL-8 secretion (data not shown).

Alongside the establishment of either the anti- or proinflammatory profile of the 158 strains using the HT-29 model, we then determined their ability to modulate IL-12p70 and IL-10 secretion by PBMCs. The IL-12p70/IL-10 ratio allows us to classify strains with a strong or a weak proinflammatory profile, with a high versus low IL-12p70/IL-10 ratio, respectively. As shown in Fig. 3, the 158 tested strains again displayed different immunomodulatory profiles after coincubation with PBMCs, confirming partially the results obtained with the HT-29 model.

Selection of the most interesting candidate probiotic strains. In order to determine the potential probiotic effect of the most interesting strains, 3 different responsive strains (Fig. 2 and 3, arrows) were chosen for further *in vivo* experiments: one with a highly proinflammatory profile (*L. plantarum* CNRZ1997), one with a weakly proinflammatory profile (*L. brevis* VEL12208), and a third with a markedly anti-inflammatory profile (*L. paracasei* VEL12195). We first confirmed the immunomodulatory profile of the 3 selected strains by calculating the IL-12p70/IL-10 ratio after coculture of the candidate strains with PBMCs from 5 different donors. As expected, the results obtained with the 5 different donors confirmed the profile of each strain and clearly showed a significant difference between anti- and proinflammatory strains (Fig. 4).

***In vivo* effects of selected strains in a murine model of IAV infection.** The effects of the 3 selected strains in a murine model of influenza virus infection were then investigated (Fig. 5). Ten days after virus challenge (2,000 PFU/mouse), the area under the weight curve (AUWC), representative of the cumulative weight loss of mice over the time course of the assay, revealed severe injury to PBS-treated mice (Fig. 6A). Mice treated with the proinflammatory strain CNRZ1997 showed the highest AUWC value compared to nontreated mice or mice treated with either a weakly proinflammatory (VEL12208) or anti-inflammatory (VEL12195) strain. Surprisingly, no significant differences were observed between nontreated mice and mice treated with either the *L. casei* DN114-001 or *L. rhamnosus* GG positive-control strain (data not shown).

These promising results demonstrate a tendency of *L. plantarum* strain CNRZ1997 to decrease the body weight loss after infection with a high dose of IAV (i.e., 2,000 PFU/mouse). We then decided to test the probiotic effects of strain CNRZ1997 with a lower dose of IAV (100 PFU/mouse). This lower virus dose was used to induce moderate symptoms in mice, which can be significantly counterbalanced by our candidate probiotic bacterium.

PBS-treated and infected mice displayed a weight loss of ~30% (at day 10) compared to healthy control mice, while mice treated with proinflammatory *L. plantarum* strain CNRZ1997 displayed a reduction in weight loss of only ~10% of body weight (at day 10). In addition, weight loss was delayed in CNRZ1997-treated mice over the time course of the assay, and an earlier initiation of recovery was also observed than for PBS-treated mice (data not shown). These results were confirmed by AUWC analysis (Fig. 6B).

To better characterize the beneficial effects of strain CNRZ1997 in the murine model of IAV infection, we used a five-point scale of visual scores. At day 0, the 3 groups of mice showed a score of 5 (Fig. 7A). At day 10, PBS-treated and infected mice presented a score of 3 (moderate symptoms), while mice treated with strain CNRZ1997 showed a score of ~4.

Influenza virus titers in infected mice. Virus titration in the lungs of infected mice revealed that CNRZ1997-treated mice presented a lower virus titer than infected control mice at days 10 and 14 postinfection (Fig. 7B). However, at day 10, no significant difference

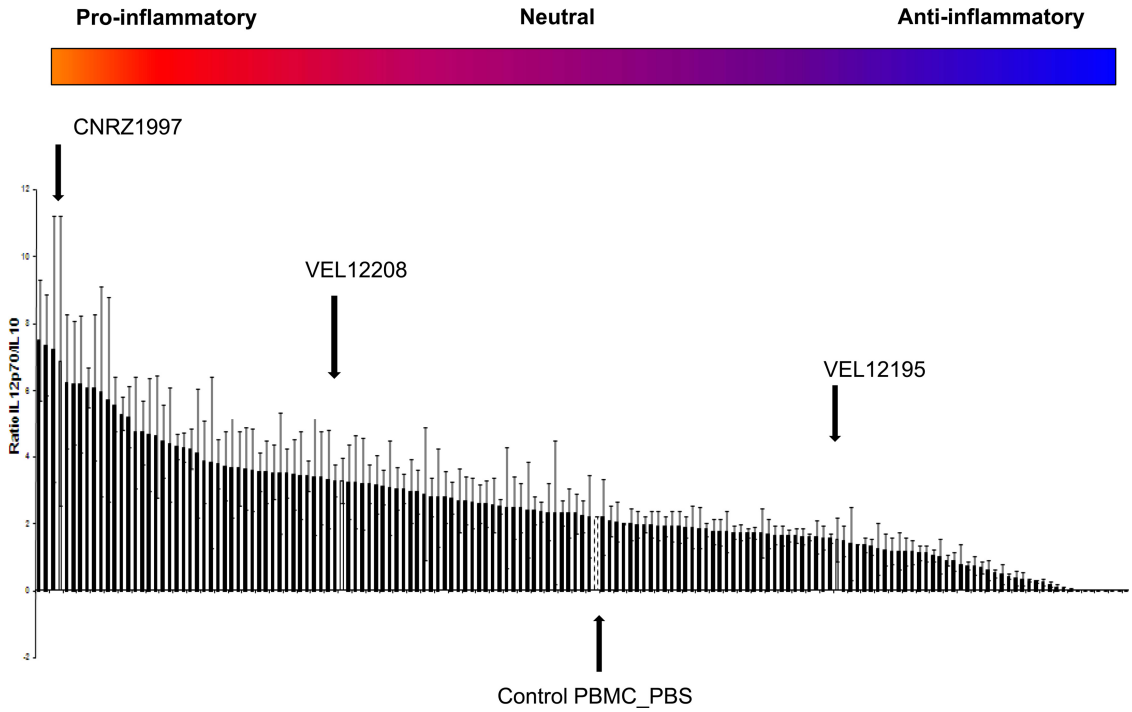

FIG 3 IL-12p70/IL-10 ratio after incubation of bacteria with PBMCs from one donor. Cytokines were quantified by ELISA after coincubation of bacteria and PBMCs for 24 h. Ratios of IL-12p70/IL-10 ± standard errors of the means are shown ($n = 3$).

was observed in virus loads between control mice and mice receiving strain CNRZ1997; this difference became significant at day 14.

DISCUSSION

Despite advances in medicine, RVI (such as the common cold or flu) continue to cause a considerable economic burden; fortunately, some probiotic strains have been studied for their positive effects on certain infectious diseases, and in the last 5 years, their use to prevent and treat RVI has significantly increased (11, 12). There is thus a clear interest in the identification and characterization of new candidate strains with well-demonstrated probiotic properties against RVI.

In this study, we determined the immunomodulatory properties of 158 strains of LAB in order to identify the most interesting candidate probiotic strain able to alleviate RVI symptoms. For this, we first performed a large-scale screening of the 158 strains for their resistance to bile salts. Indeed, bile salts may constitute a deleterious factor preventing a given strain from exerting its beneficial properties *in vivo*. A high-throughput method was used to rapidly characterize strain resistance. Among the tested strains, *L.*

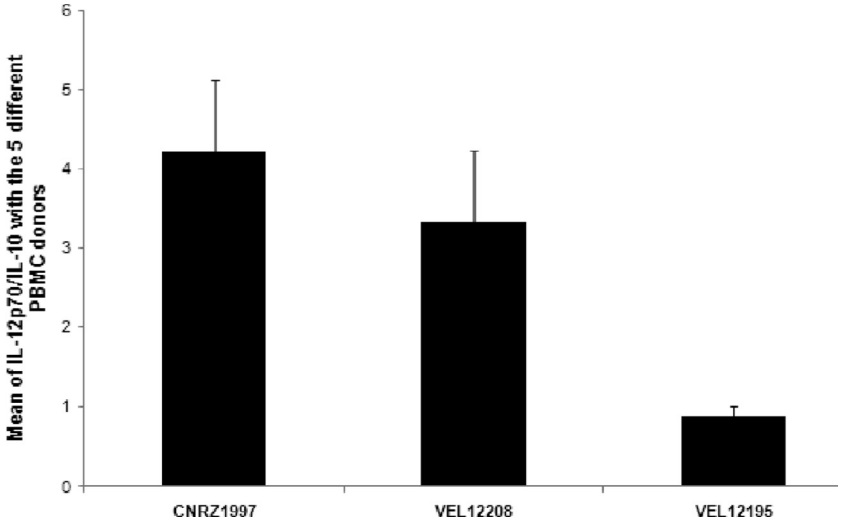

FIG 4 IL-12p70/IL-10 ratios for the selected strains with 5 different PBMC donors. Cytokines were quantified by ELISA after coincubation of bacteria and PBMCs for 6 h. Ratios of IL-12p70 to IL-10 ± standard errors of the means are shown ($n = 3$).

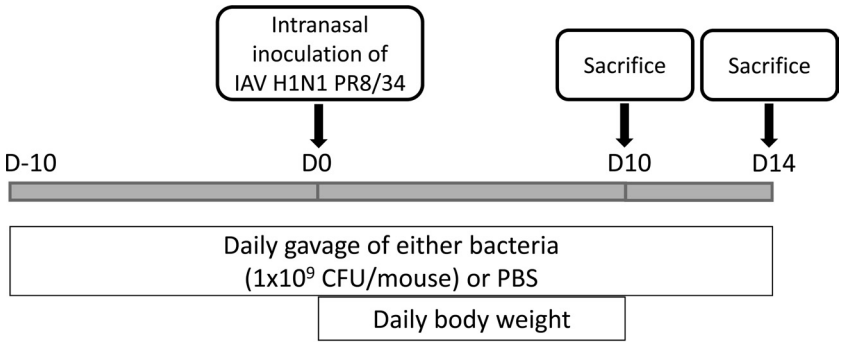

FIG 5 Protocol used to study the immunomodulatory effect of selected bacteria on mice infected with the A/PR8/34 strain (a mouse-adapted strain).

plantarum CNRZ1997 had a delay of 2.9 h. Eighty strains (62.5% of the collection), including VEL12208 and VEL12195, showed moderate resistance, with a growth delay of between 5 and 13 h. Eight strains were not resistant to bile salts. This simple yet robust protocol could also be used to test other properties, such as other stresses or the ability to grow under specific conditions.

The immunomodulatory effects of the bacterial collection were then assessed by using two cellular models: TNF-α-activated HT-29 cells and peripheral blood mononuclear cells (PBMCs). These *in vitro* cellular models are commonly used to validate the immunomodulatory properties (anti- or proinflammatory cytokine profile) of a given candidate bacterium before validation in animal models. Indeed, these tests have a predictive value and allow a reduction of the number of bacterial strains to be tested in animal models, which are expensive and time-consuming. One of these predictive *in vitro* models is PBMCs, in which the immunomodulation potential of probiotic strains is usually assessed by measuring IL-10 and IL-12 cytokine levels after stimulation with live bacterial strains. In this manner, Foligne et al. (9) successfully established a correlation between this PBMC model, the *in vivo* immunomodulation potential of probiotic strains, and the ability to prevent experimental colitis in mice. Bacteria inducing higher levels of the anti-inflammatory cytokine IL-10 and lower levels of the proinflammatory cytokine IL-12 *in vitro* displayed the best protection in the murine colitis model (15). The human intestinal epithelial cell line HT-29 has been also used successfully to evaluate the immunomodulatory properties of bacteria (25, 26). Once coincubation with bacterial strains was achieved, IL-8 quantification led to the selection of anti-inflammatory bacteria (reducing IL-8 production) and proinflammatory bacteria (enhancing IL-8 production) (25, 27). After we tested our bacterial collection with these two cellular models, we selected 3 different responsive strains according to their immunomodulatory profile, one with a highly proinflammatory profile (*L. plantarum* CNRZ1997), one

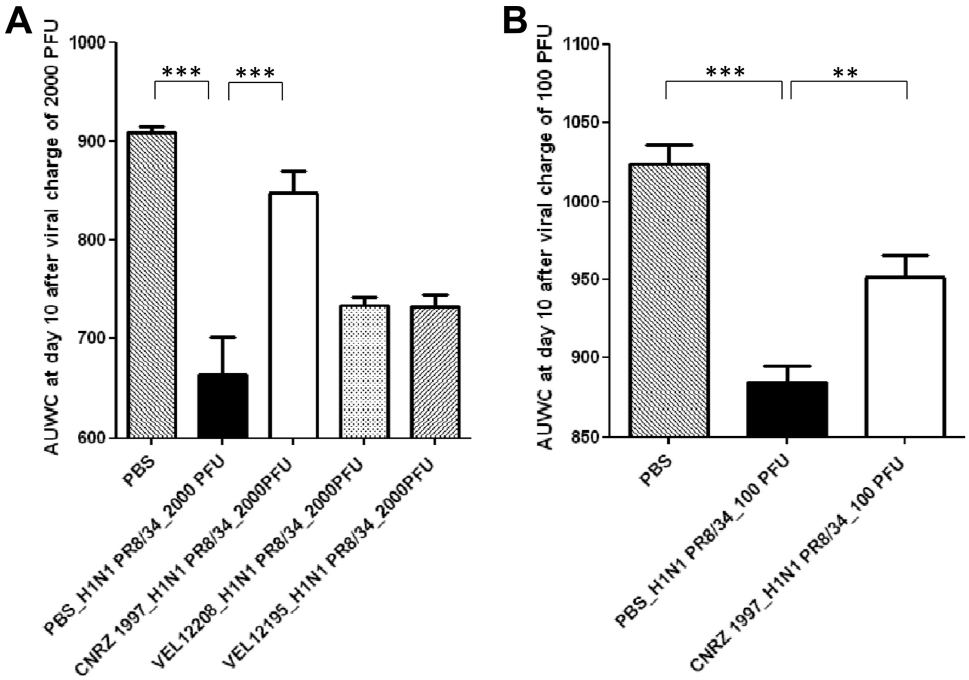

FIG 6 (A) Area under the weight curve (AUWC) for the different groups after a severe virus infection with 2,000 PFU of the A/PR8/34 strain. Statistically significant differences were determined by Dunnett's test (***, $P < 0.001$). (B) Area under the weight curve for the different groups after a moderate viral infection with 100 PFU of A/PR8/34 strain. Statistically significant differences were determined by Dunnett's test (***, $P < 0.001$; **, $P < 0.01$).

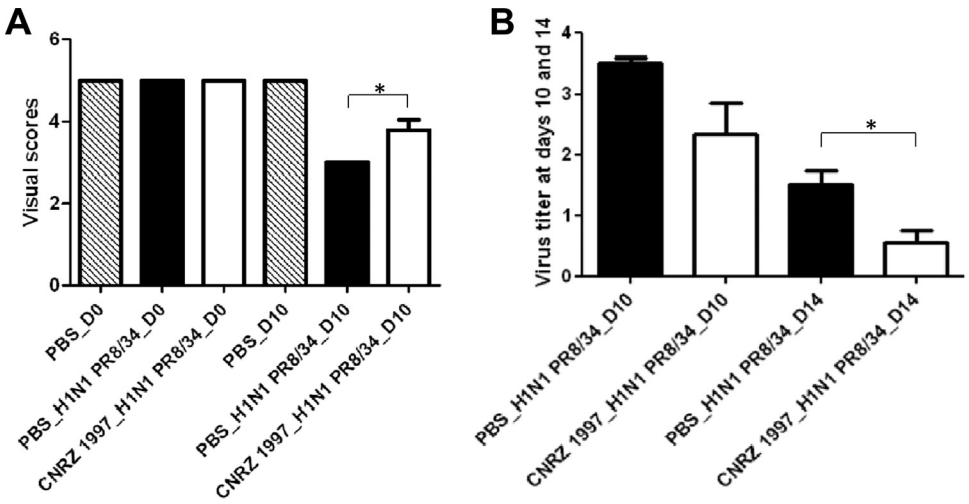

FIG 7 (A) Visual score for mice after treatment with *L. plantarum* CNRZ1997 after virus infection with 100 PFU of the A/PR8/34 strain. Statistically significant differences were determined by Dunnett's test (∗, $P < 0.05$). (B) Virus titer in lungs at day 10 after virus infection. Results are expressed as the log number of copies of the virus genome in 2.5 μg of total RNA. Statistically significant differences were determined by Student *t* test (∗, $P < 0.05$).

with a weakly proinflammatory profile (*L. brevis* VEL12208), and one with a markedly anti-inflammatory profile (*L. paracasei* VEL12195), for *in vivo* experiments using a model of A/PR8/34 strain infection in mice. Based on the preliminary results observed with a high dose of IAV (2,000 PFU), we found that *L. plantarum* strain CNRZ1997 (highly proinflammatory profile) was the most interesting candidate among the three candidate strains tested, with beneficial effects against IAV. The results obtained with a less challenging model (lower dose of IAV of 100 PFU) as well as with the five-point scale of visual scores confirmed these observations. Indeed, this strain was effective in preventing body weight loss (Fig. 6B) and clinical condition alterations by alleviating significantly the symptoms of the infected mice ($P < 0.05$) (Fig. 7A). Kawase et al. (28) previously found similar results (i.e., improved clinical symptoms) when studying the oral administration of two LAB strains, *L. rhamnosus* GG and *L. gasseri* TMC0356, in a murine model of IAV infection.

In addition, when we analyzed virus titration in lungs of infected mice at 10 and 14 days postinfection, we found that strain CNRZ1997 was able to decrease virus titers in these infected mice. This result indicates a positive effect of strain CNRZ1997 in mice by perhaps accelerating IAV elimination from the lungs. These results are similar to those obtained previously by Kawashima et al. (29), where *L. plantarum* strain LpYU (with a proinflammatory profile observed *in vitro*) was tested in a model of virus infection with the H1N1 virus (A/NWS/33).

In this work, we decided to use strains GG and DN114-001 as positive controls in our model of IAV, since they have already been successfully studied for their anti-IAV properties in preclinical trials (23) and in a human clinical trial against RVI (13). Interestingly, the AUWC results observed for mice treated with either GG or DN114-001 showed that there are no protective effects of these two well-known probiotic strains in our IAV model. These unexpected results can be explained by the fact that strain GG was reported previously to be protective against IAV in mice after "intranasal" administration, in contrast with our study, where we used an "intragastric" route. In order to confirm this, we performed experiments with *L. rhamnosus* GG by the intranasal route in our IAV model, and we confirmed a protective effect; however, we cannot favor this route, since the aim of this study was to develop a probiotic oral dietary supplement. Second, most trials using strain DN114-001 against IAV have been done with the fermented final product (which means that it contained *L. casei* strain DN114-001 combined with two cultures commonly used in yogurt, *Streptococcus thermophilus* and *Lactobacillus bulgaricus*), while in our study, we used DN114-001 as a single isolated strain, so we cannot discard a synergistic effect of strain DN114-001 and the fermented final product.

The effects of probiotic bacteria against infectious diseases, such as IAV, have been demonstrated, but the mechanisms of action are not yet fully understood; however, some hypotheses have been advanced. For example, oral administration of acidic exopolysaccharide extracts of *L. delbrueckii* OLL1073R-1 to mice infected with IAV provided protection (30). In another study, the protective properties of *L. plantarum* strain LpYU against IAV were dependent on Toll-like receptor 2 (TLR2), which recognizes the peptidoglycan (PG) and the lipoteichoic acids (LTA) of Gram-positive bacteria (29). These two results seem interesting to explore in order to identify the mechanisms of action of our proinflammatory strain CNRZ1997.

Most of the studies using LAB to prevent and treat IAV infection have been performed with intranasal administration of either live or heat-killed bacteria (15, 31, 32). In this study, we chose to test live bacteria administered orally, in order to develop a potential probiotic supplement for use in humans. Of note, parallel research demonstrated that the proinflammatory *L. plantarum* strain CNRZ1997 is compatible with all manufacturing and formulation technological processes (data not shown), and currently, a double-blind, randomized, controlled trial is in progress in order to investigate whether the consumption of this strain influences the severity of symptoms and the incidence and duration of common cold infections. In conclusion, our results suggest the feasibility of a large *in vitro* screening of bacterial strains using two cellular models (TNF-α-activated HT-29 cells and PBMCs), in order to determine their immunomodulatory properties based on a cytokine profile. Oral administration of the most proinflam-

matory strain (*L. plantarum* CNRZ1997) conferred protection against IAV infection. Further research is being conducted in our laboratory to identify the mechanism of action of this new candidate probiotic bacterium.

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